

The interaction of titin and α -actinin is controlled by a phospholipid-regulated intramolecular pseudoligand mechanism

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The assembly of stable cytoskeletal structures from dynamically recycled molecules requires developmental and spatial regulation of protein interactions. In muscle, titin acts as a molecular ruler organizing the actin cytoskeleton via interactions with many sarcomeric proteins, including the crosslinking protein α -actinin. An interaction between the C-terminal domain of α -actinin and titin Z-repeat motifs targets α -actinin to the Z-disk. Here we investigate the cellular regulation of this interaction. α -actinin is a rod shaped head-to-tail homodimer. In contrast to C-terminal fragments, full-length α -actinin does not bind Z-repeats. We identify a 30-residue Z-repeat homologous sequence between the actin-binding and rod regions of α -actinin that binds the C-terminal domain with nanomolar affinity. Thus, Z-repeat binding is prevented by this 'pseudoligand' interaction between the subunits of the α -actinin dimer. This autoinhibition is relieved upon binding of the Z-disk lipid phosphatidylinositol-bisphosphate to the actin-binding domain. We suggest that this novel mechanism is relevant to control the site-specific interactions of α -actinin during sarcomere assembly and turnover. The intramolecular contacts defined here also constrain a structural model for intrasterical regulation of all α -actinin isoforms.

Keywords: α -actinin/connectin/phosphatidylinositides/phosphatidylinositide kinases/titin

Introduction

The cytoskeleton consists of a number of filamentous systems, composed of polymers of actin, tubulin or intermediate filament proteins. These form the filaments of actin stress fibres, microtubules or intermediate filaments. While their filamentous state provides the cell with an internal scaffold, maintaining cell shape and providing routes for intracellular traffic, the dynamic exchange of subunits and the linking of the different systems by special crosslinking proteins allows the cell to respond rapidly to altered mechanical needs. In muscle cells, the cytoskeleton is dominated by actin filaments arranged in contractile units, the sarcomeres. In the sarcomeric Z-disks, anti-parallel actin filaments overlap and are crosslinked in a tetragonal lattice by the ubiquitous actin-crosslinking

protein α -actinin (Luther, 2000). The number of α -actinin crosslink levels in the Z-disk is tightly regulated in a muscle-specific way by differentially spliced binding sites, called Z-repeats (Gautel *et al.*, 1996; Figure 1), on the giant ruler protein titin (also known as connectin; see review by Maruyama, 1997). The resulting lattice is of great mechanical stability, as it bears both the passive and active strain that arises when muscle is passively stretched, or actively contracts (Vigoreaux, 1994). However, during the embryonic generation of muscle, as well as during the formation of new sarcomeres in pre-existing muscle (e.g. during cardiac hypertrophy), the Z-disk forms the dynamic starting point of myofibrillogenesis (Tokuyasu and Maher, 1987; Fürst *et al.*, 1989; Schultheiss *et al.*, 1990; Rhee *et al.*, 1994). New α -actinin and actin subunits are then incorporated, and this has to happen in a spatially and temporally controlled way that leads to a remarkably high degree of order not found to the same extent in other actin-based cytoskeletal structures.

α -actinin is a major F-actin crosslinking protein in both muscle and non-muscle cells (Blanchard *et al.*, 1989). It is a homodimeric rod-shaped molecule with an actin-binding domain at each end (Figure 1). Several α -actinin isoforms exist that are regulated differently: actin binding in the non-muscle isoforms is Ca^{2+} sensitive, whereas in muscle α -actinins it is not. Apart from its interaction with actin, α -actinin has emerged as a major multivalent platform mediating interactions with many cytoskeletal or regulatory proteins. At the membrane, in focal adhesions and cell-cell contacts, α -actinin interacts with capZ, vinculin, zyxin and cell surface receptors such as the NMDA or integrin receptors (Wachsstock *et al.*, 1987; Otey *et al.*, 1990; Carpen *et al.*, 1992; Crawford *et al.*, 1992; McGregor *et al.*, 1994; Heiska *et al.*, 1996; Beckerle, 1997; Papa *et al.*, 1999). Along the actin cytoskeleton, α -actinin interacts with signalling molecules such as the protein kinases MEKK1 and PKN (Mukai *et al.*, 1997; Christerson *et al.*, 1999), zinc-finger proteins like ALP (Crawford *et al.*, 1992; Pomiès *et al.*, 1997) and PDZ-domain proteins like ZASP/cypher (Xia *et al.*, 1997; Faulkner *et al.*, 1999; Pomiès *et al.*, 1999; Zhou *et al.*, 1999). In muscle, in addition to some of these proteins, the interactions with sarcomeric proteins are possibly the most important functionally. α -actinin binds to the sarcomeric ruler proteins titin and nebulin (Nave *et al.*, 1990) and to the novel Z-disk proteins myotilin (Salmikangas *et al.*, 1999), ZASP (Faulkner *et al.*, 1999) and FATZ (Faulkner *et al.*, 2000). Although most of these interactions appear to be with the spectrin-like repeats (e.g. myotilin, FATZ, titin, NMDA receptor), the CaM-like domain is also emerging as an interaction domain for diverse proteins, e.g. the Z-disk-associated PDZ-domain protein ZASP (Faulkner *et al.*, 1999) or the LIM-protein hCLIM1 (Kotaka *et al.*, 2000). The mechanism by which

interactions with the CaM-domain are regulated is, therefore, of general importance for understanding the diverse ligand interactions of α -actinin in the actin cytoskeleton. The interaction with the giant ruler protein (Trinick, 1996) titin seems to be primarily responsible for targeting α -actinin specifically to the Z-disk, and specifies the number of crosslinks between overlapping actin filaments in this structure. However, for most of these interactions, relatively little is known about the exact binding sites between both proteins and even less about the cellular regulation mechanisms that control them. The interaction with titin is amongst the best characterized, and therefore can serve as a model system for the study of the control mechanisms governing several other types of α -actinin interactions.

The discovery of two classes of α -actinin binding sites in the Z-disk portion of titin was a first step towards a molecular understanding of the mechanisms of protein sorting in the muscle actin cytoskeleton. Titin Z-repeats provide multiple binding sites for the C-terminal calmodulin-like (CaM) domain of α -actinin (Ohtsuka *et al.*, 1997a,b; Sorimachi *et al.*, 1997; Young *et al.*, 1998). Alternative splicing of the Z-repeats appears to correlate with variations in Z-disk thickness (Gautel *et al.*, 1996). At the Z-disk periphery, a unique site interacts with the two central spectrin-like repeats of the α -actinin rod (Young *et al.*, 1998). Whereas this site is active both in truncated fragments of α -actinin as well as in the full-length molecule, all previous reports on α -actinin-Z-repeat interactions refer to α -actinin fragments lacking their N-terminal domains, or used titin fragments containing part of the rod-binding domain. In contrast, binding assays with native full-length α -actinin failed to detect an interaction with Z-repeats (Gautel *et al.*, 1996). Although such negative findings may be explained by intrinsic problems of the interaction assays used, they may also reflect a genuine property of the full-length molecule. The controlled activation of protein-protein interactions seems to be a major prerequisite in order to achieve the sequential integration of subunits into the nascent sarcomere observed *in vivo*. Therefore, we investigated which elements in α -actinin are required to control the interaction with Z-repeats, and which external factors are involved in their modulation.

Results

Binding of titin Z-repeats to the CaM-like domain is inhibited in the intact α -actinin dimer

The interaction between titin Z-repeats and the CaM-like domain of α -actinin was found by screening with titin fragments as baits in the yeast two hybrid system. (Ohtsuka *et al.*, 1997a,b; Sorimachi *et al.*, 1997). In these experiments, none of the positive clones obtained encoded a full-length α -actinin molecule. More particularly, all clones began in the rod domain of α -actinin, none contained any of the actin-binding domain (ABD). Also, the *in vitro* binding of the central Z-repeats that we observed previously was to an α -actinin construct (R1-C) lacking the actin-binding domain (Young *et al.*, 1998). Therefore, using the yeast two hybrid system and *in vitro* binding assays, we tested whether a full-length α -actinin could interact with titin Z-repeats.

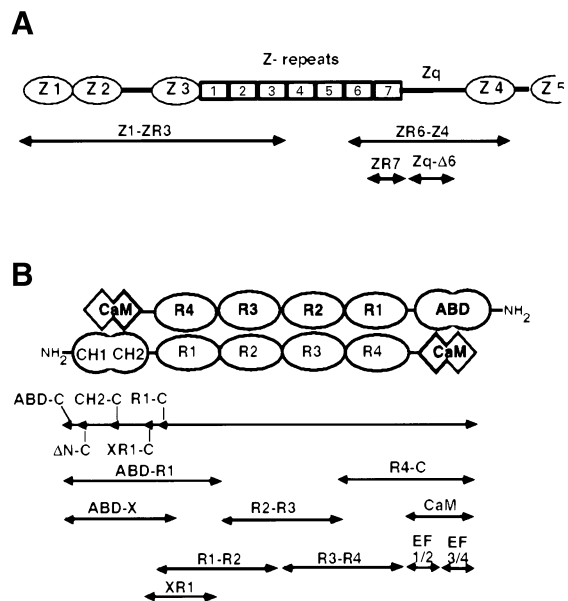


Fig. 1. (A) Domain structure of the Z-disk region of titin. Z1, Z2, Z3 and Z4 = immunoglobulin-like domains; ZR1–ZR7 = Z-repeats; Zq = non-modular region. Only ~100 kDa of the 3 MDa titin molecule are shown. (B) Schematic diagram of the α -actinin anti-parallel dimer showing the domain structure of the molecule. The N-terminal actin-binding domain is composed of two calponin homology domains. The central rod region consists of four spectrin-like repeats. A CaM-like domain is located at the C-terminus of the molecule. ABD = actin binding domain; CH = calponin homology domain; R1, R2, R3 and R4 = spectrin-like repeats; CaM = calmodulin-like domain. Shown below each molecule are the constructs used in this study. See Materials and methods for the amino acid sequence of these constructs. The nomenclature indicates the most N- and C-terminal domains in a given construct. '-C', constructs that contain the C-terminus of the molecule.

Interactions between the titin fragments Z1-ZR3 or zq-Z4 and various α -actinin constructs were tested in the yeast two hybrid system (Figure 2A and B, part I). zq-Z4 binds to the central spectrin repeats of the α -actinin rod (Young *et al.*, 1998) while Z1-ZR3 was known to interact with truncated α -actinins such as R1-C, presumably through the Z-repeats. In contrast, we now found that the full-length α -actinin, while it binds to zq-Z4, does not show any interaction with Z1-ZR3. The interaction with zq-Z4 demonstrates that the full-length construct is functional in the yeast two-hybrid system, i.e. it is expressed and transported to the yeast nucleus. We concluded, therefore, that upon dimerization some part of the α -actinin ABD was inhibiting Z-repeat binding. Deletion of the first CH domain of the ABD in the CH2-C construct did not relieve this inhibition. Inspection of the α -actinin amino acid sequence identified a region between the second CH domain and the first repeat of the rod that shows some homology to the titin Z-repeats (Figure 3) and that might, therefore, interact with the CaM-like domain in an analogous manner. A construct, XR1-C, was designed, which starts 15 residues N-terminal from the start of R1, and was found not to interact with Z1-ZR3. This suggested that a region of α -actinin between the ABD and R1 was capable of inhibiting the binding of Z-repeats in intact α -actinin molecules.

In order to confirm these yeast two hybrid results in an *in vitro* binding assay, titin ZR7, shown to be a strongly

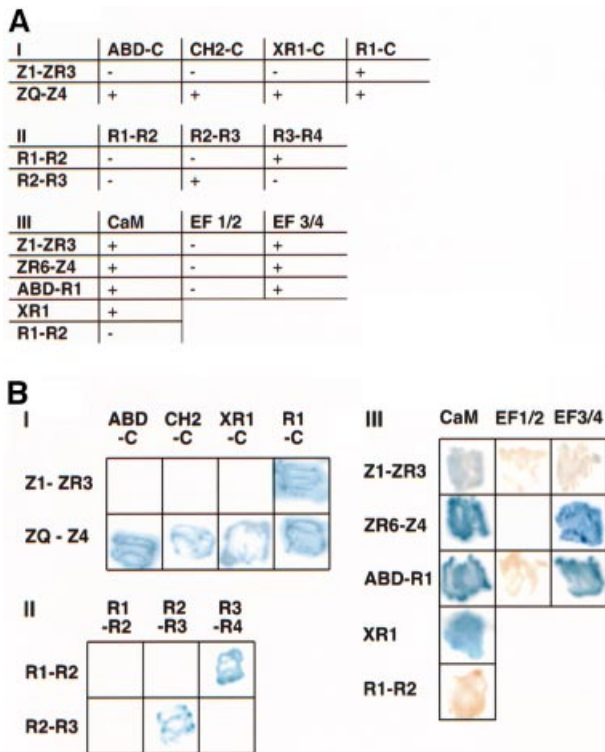


Fig. 2. Yeast two-hybrid analysis of the intersubunit and titin interactions of the α -actinin dimer. α -actinin-2 and titin constructs were cloned into the pLexA or pGAD10 vectors and cotransformed into the reporter yeast strain. Interactions between constructs, as shown by expression of the His3 and β -galactosidase reporter genes, was monitored by scoring growth on plates lacking histidine (A) or in colorimetric filter based assays using X-gal as substrate (B). pLexA constructs are shown on the left and pGAD10 constructs on the top of each grid/table. (I) Interactions between titin constructs (Z1-ZR3 and ZQ-Z4) and N-terminally truncated α -actinin molecules. See Figure 1 for details of the nomenclature of the constructs. (II) Interactions between pairs of repeats from the α -actinin rod. (III) Interactions of the α -actinin CaM-like domain with titin constructs containing Z-repeats and with the α -actinin ABD-R1 construct. Division of the CaM domain into two halves (EF1/2 and EF3/4) shows that both binding activities are mediated by EF-hands 3/4. An R1 construct, XR1 extended at the N-terminus by 15 residues, is sufficient to mediate the interaction with the CaM domain.

binding Z-repeat (Ohtsuka *et al.*, 1997b; Sorimachi *et al.*, 1997), was expressed as a glutathione S-transferase (GST) fusion protein (Figure 4). Various α -actinin constructs were expressed as His₆-tagged proteins in the pET8c-6HTEV vector (Figure 4). The His₆ tag was removed by TEV protease digestion to prevent possible interference in the binding assays. Binding of the α -actinin constructs to GST-ZR7 was assayed in a pull-down experiment using mini glutathione-Sepharose columns (Figure 5). The results completely confirmed the yeast two-hybrid data. Thus, the full-length α -actinin did not bind; neither did N-terminal deletion constructs with either the isoform-specific first 18 residues or the first CH domain deleted. The R1-C construct did bind but N-terminal extension of R1 by 15 residues inhibited Z-repeat binding.

The repeats of the α -actinin rod dimerize in an aligned manner

The α -actinin rod contains four spectrin-like repeats that are thought to be responsible for the dimerization of the

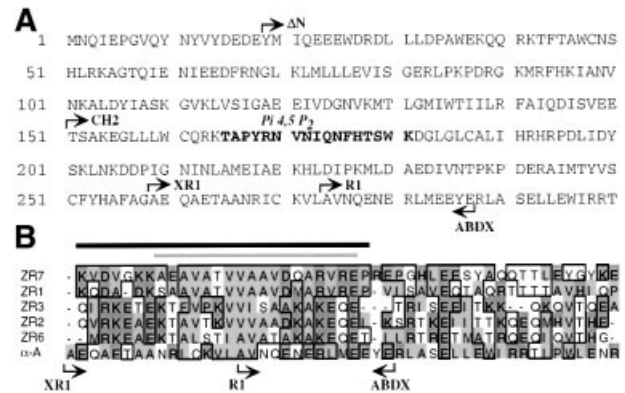


Fig. 3. (A) Sequence of the first 300 amino acids of skeletal muscle α -actinin 2A. N-termini and C-termini of the various constructs used are indicated. Highlighted in bold is the P14,5P₂ binding site as identified by Fukami *et al.*, (1996). (B) Sequence alignment of five of the titin Z-repeats (numbered according to Sorimachi *et al.*, 1997) with a region of α -actinin (α -A) that spans the C-terminal end of the actin-binding domain and the beginning of the first repeat of the rod. Homologous residues are boxed, non-polar residues are shaded light grey, charged residues are shaded dark grey. Note the hydrophobic cluster of residues in the first half of the alignment, which is the hallmark of the Z-repeats. α -actinin residues 259–307 can be aligned with the Z-repeats and share a similar hydrophobic region as well as some other conserved residues. Positions of the N- and C-termini of various constructs are indicated. The dark bar indicates the α -actinin binding site within ZR7 defined by Ohtsuka *et al.* (1997b). The light bar indicates the region of ZR7 that is helical when in complex with the α -actinin EF3/4 region (Atkinson *et al.*, 2000).

molecule. In order to gain an understanding of how these repeats as well as the N-terminal ABD and the C-terminal domain are arranged in the α -actinin dimer, the yeast two-hybrid system was used to investigate interactions between the repeats. Double repeat constructs (R1R2, R2R3 and R3R4) were cloned into the pLex and pGAD10 vectors and interactions between them were monitored by activation of the β -galactosidase and His3 reporter genes (Figure 2A and B, part II). R1R2 and R3R4 were found to interact with each other while neither could interact with the R2R3 construct. The R2R3 construct was found to interact with itself. These results are consistent with an aligned arrangement of the repeats in the α -actinin dimer as shown Figure 1. If the rod domains in the dimer were staggered by one repeat in either direction then R2R3 would be positioned directly opposite either R1R2 or R3R4, depending on the direction of the stagger. No interactions are observed between these pairs of repeats, arguing strongly in favour of an aligned arrangement that would bring the actin-binding domain and CaM-like domain into close proximity.

In α -actinin, a region between ABD and R1 acts as a pseudo-Z-repeat in interacting with the CaM-like domain

The alignment shown in Figure 3 identifies a region between residues 260 and 300 of α -actinin that shows homology to titin Z-repeats, and inclusion of this region in the XR1-C construct is sufficient to inhibit binding of Z-repeats to the CaM-like domain. This inhibition of Z-repeat binding might be caused by either a specific competitive interaction of this region with the CaM-like

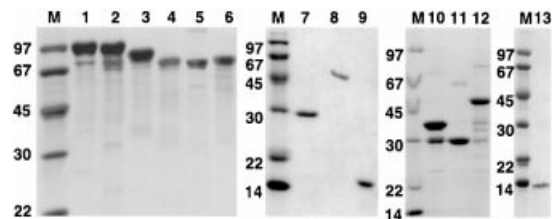


Fig. 4. Proteins used in this study. Recombinant proteins were expressed in *E.coli* as described and analysed by SDS-PAGE with Coomassie Blue staining. Calculated molecular weights (in kDa) are shown in brackets. Lanes 1–9: α -actinin constructs (2A isoform unless stated otherwise). Lane 1: ABD-C (104); lane 2: Δ N-C (102); lane 3: CH2-C (87); lane 4: XR1-C (74); lane 5: R1-C (73); lane 6: α -actinin-1A R1-C (73); lane 7: R4-C (31); lane 8: ABD-R1 (46); lane 9: CaM-like domain (17). Lanes 10–13: titin constructs. Lane 10: GST-ZR7 (34); lane 11: GST (29); lane 12: GST-Zq- Δ 6 (43); lane 13: ZR7 (6). M, molecular weight markers (sizes shown in kDa). Note that the GST-ZR7 fusion protein shows slight degradation. The lower band is GST that has lost the ZR7 fragment during purification.

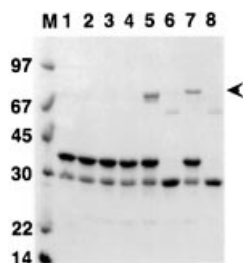


Fig. 5. Binding of α -actinin constructs to GST-ZR7. Proteins were mixed and bound to GST affinity beads as described. Proteins that remained bound after washing were eluted with loading buffer and visualized by SDS-PAGE with Coomassie Blue staining. Lane 1: α -actinin-2 ABD-C/GST-ZR7; lane 2: α -actinin-2 Δ N-C/GST-ZR7; lane 3: α -actinin-2 CH2-C/GST-ZR7; lane 4: α -actinin-2 R1ext-C/GST-ZR7; lane 5: α -actinin-2 R1-C/GST-ZR7; lane 6: α -actinin-2 R1-C/GST; lane 7: α -actinin-1 R1-C/GST-ZR7; lane 8: α -actinin-1A R1-C/GST. M, molecular weight marker. Note, a faint band of 60 kDa in the GST control lanes probably represents some dimeric GST. Bound α -actinin R1-C is indicated by the arrowhead.

domain, or simply by a steric blocking of the Z-repeat binding site by this region upon formation of the α -actinin dimer. In order to distinguish between these possibilities, we looked for an interaction between the isolated CaM-like domain and the construct ABD-R1, which includes the ABD plus the first repeat of the rod. Binding of the CaM-like domain to ABD-R1 as well as to the titin Z-repeat containing constructs Z1-ZR3 and ZR6-Z4 was observed in yeast two-hybrid assays (Figure 2A and B, part III). The construct XR1, which includes all of the Z-repeat homologous region at the N-terminus of R1, was sufficient to mediate this interaction. No binding of the CaM-like domain to R1R2 was observed, which excludes the possibility that the interaction with ABD-R1 is a non-specific interaction with the surface of R1 that would form the dimer interface in the intact α -actinin. When the CaM-like domain is split in half, the second half, comprising EF-hands 3 and 4, is sufficient for interaction with Z-repeats as well as with ABD-R1, suggesting that both bind in an analogous manner.

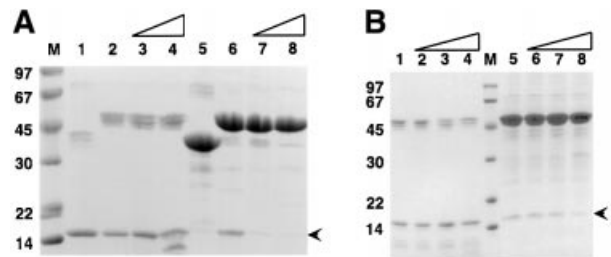


Fig. 6. (A) Competitive binding of α -actinin-2 ABD-R1 and titin ZR7 to the α -actinin-2 CaM-like domain (CaM). Proteins were mixed and bound to Ni-NTA agarose as described and the fractions analysed by SDS-PAGE with Coomassie Blue staining. The flow-through and wash fractions were mixed and loaded in lanes 1–4. Bound proteins were eluted with loading buffer and run in lanes 5–8. Lanes 1 and 5: His₆-A BDX/CaM; lanes 2 and 6: His₆-ABD-R1/CaM; lanes 3 and 7: His₆-ABD-R1/CaM/20 μ M ZR7; lanes 4 and 8: His₆-ABD-R1/CaM/100 μ M ZR7. The arrowhead indicates the bound CaM-like domain. ZR7 stains poorly by Coomassie and is only visible at the highest concentration used (lane 4). (B) An α -actinin peptide comprising amino acids 259–287 inhibits binding of ABD-R1 to the CaM-like domain. The α -actinin His₆-ABD-R1 and CaM constructs are mixed with increasing concentrations of peptide and bound to Ni-NTA agarose as in (A). After washing, bound proteins are eluted with loading buffer. Lanes 1–4: flow-through and wash fractions mixed; lanes 5–8: eluate fractions. Lanes 1 and 5: no peptide; lanes 2 and 6: 30 μ M peptide; lanes 3 and 7: 150 μ M peptide; lanes 4 and 8: 600 μ M peptide. The arrowheads indicate the bound CaM-like domain.

The binding of the CaM-like domain to ABD-R1 was confirmed in an *in vitro* pull-down assay (Figure 6A). Addition of ZR7 could outcompete the binding of the CaM-like domain to ABD-R1 in this assay. ABDX, a construct that includes most of this Z-repeat homologous region, was unable to bind the CaM-like domain in the same assay, suggesting that either the Z-repeat homologous region is insufficient for binding or that R1 is required for stabilization of this region. Isothermal calorimetry was used to determine the relative strength of the interactions of ZR7 and ABD-R1 with the CaM-like domain (Figure 7). A K_d value of 186 nM was calculated for ZR7 compared with 568 nM for ABD-R1. R4-C bound to ABD-R1 with higher affinity ($K_d = 215$ nM) and enthalpy than the CaM-like domain alone, reflecting the additional interactions between the spectrin-like repeats R1 and R4.

A peptide comprising most of the Z-repeat homologous region (259–287) of α -actinin was synthesized and tested for its ability to outcompete the binding of the CaM-like domain to ABD-R1. Some reduction of binding was seen at higher concentrations of peptide (Figure 6B); however, a circular dichroism spectrum of the peptide showed a low helical content, indicating that the peptide was poorly folded on its own (not shown).

Regulation of the Z-repeat: α -actinin interaction by phospholipids *in vitro*

If intact α -actinin molecules are not able to bind Z-repeats, this calls into question the *in vivo* relevance of the interaction unless there is an activation mechanism to switch on the binding. Like several other actin-binding proteins, α -actinin has been reported to bind phospholipids (Fukami *et al.*, 1992; Niggli and Gimona, 1993) and phospholipids are known to be a component of the Z-disk (Bullard *et al.*, 1990). Therefore, the effects of various

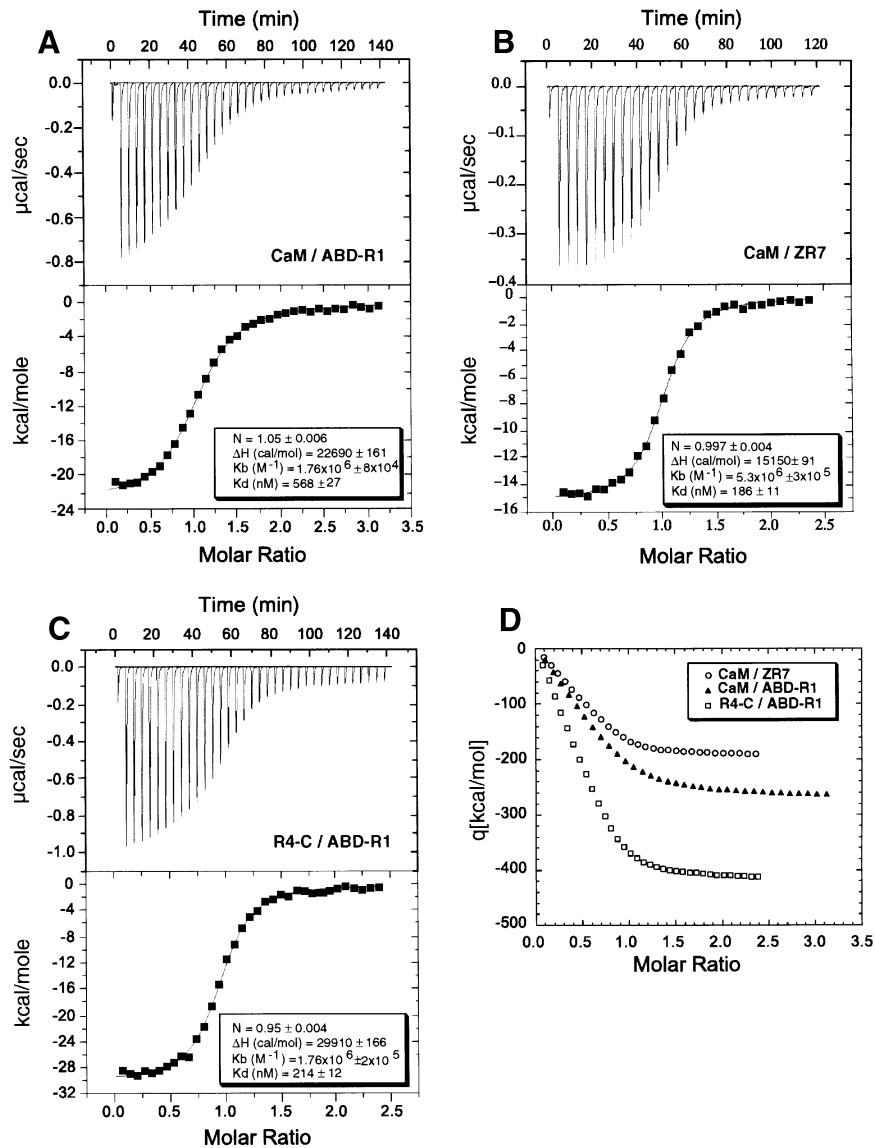


Fig. 7. Inter-subunit and titin interactions of α -actinin demonstrated by isothermal calorimetry. The α -actinin CaM-like domain (CaM) or R4-C was titrated against ABD-R1 or titin ZR7 by injections of equal volume at regular time intervals. (A) 150 μ M CaM and 10 μ M ABD-R1; (B) 90 μ M CaM and 9 μ M ZR7; (C) 215 μ M R4-C and 10 μ M ABD-R1. The top graph in each case shows the raw data; the heat of the binding reaction is monitored as the differential energy (μ cal/s) required to maintain the experimental cell at the same temperature (26°C) as the reference cell. The lower graph shows the processed data after normalization per mol of injectant, integration with respect to time and subtraction of reference data. The solid line is the calculated best fitting curve to this data obtained by optimization of the fitting parameters stoichiometry (n), enthalpy (ΔH) and association constant (K_b) using the least squares minimization routines provided in the Origin software. The values of these parameters obtained for each interaction are shown. (D) The integral mode of the data in A–C; integrated enthalpies are plotted against molar ratios of the respective proteins.

phospholipids on the Z-repeat- α -actinin interaction were tested (Figure 8). It was found that the anionic phospholipids phosphatidylserine and phosphatidylinositol 4,5-bisphosphate (Pi4,5P₂) could activate binding of full-length α -actinin to GST-ZR7 in a dose-dependent manner (Figure 8). Phosphatidylcholine had no effect. We also tested whether either of the Pi4,5P₂ head group molecules, D-myo-inositol 1,4,5-triphosphate or L- α -glycerophospho-D-myo-inositol 4,5-bisphosphate, could activate the interaction, but neither molecule showed any effect under the conditions used. A binding site for Pi4,5P₂ has been reported in the second CH domain of α -actinin (Fukami *et al.*, 1996) and binding of the phospholipid was found to regulate F-actin binding. To test which parts of the

α -actinin ABD were required for Pi4,5P₂-mediated activation of Z-repeat binding, the constructs ABD-C, CH2-C and R1X-C were used to test for Z-repeat binding in the presence of Pi4,5P₂ (Figure 8B). Z-repeat binding could only be significantly activated in the full-length α -actinin, suggesting that the entire ABD is necessary for regulation by Pi4,5P₂. Densitometrical analysis of the gels suggests \sim 90-fold activation of binding upon addition of 25 μ g/ml Pi4,5P₂.

The truncated fragment R1-C from the non-muscle α -actinin 1A isoform can also interact with Z-repeats either in yeast two-hybrid assays (not shown) or *in vitro* (Figure 5). However, in both assays the full-length protein fails to interact, although binding can be activated by

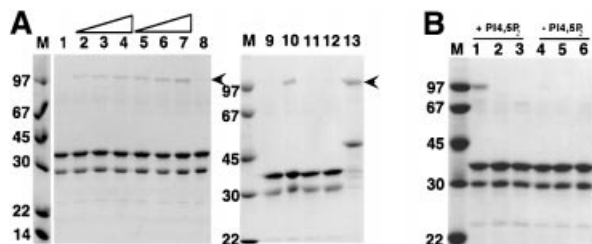


Fig. 8. (A) Effects of phospholipids on binding of full-length α -actinin to titin constructs, GST-ZR7 and GST-Zq- Δ 6. Proteins were mixed and bound to GST affinity beads as described. Proteins that remained bound after washing were eluted with loading buffer and analysed by SDS-PAGE. Phospholipids were added to protein mixture as micelles in 0.5% Triton. Lane 1: α -actinin-2 ABD-C/GST-ZR7, control with 0.5% Triton; lanes 2, 3 and 4: α -actinin-2 ABD-C/GST-ZR7, 5, 10 and 25 μ g/ml phosphatidylserine, respectively; lanes 5, 6 and 7: α -actinin-2 ABD-C/GST-ZR7, 5, 10 and 25 μ g/ml phosphatidylinositol bisphosphate (PI4,5P₂); lane 8: α -actinin-2 ABD-C/GST-ZR7, 25 μ g/ml phosphatidylcholine; lane 9: native α -actinin-1A/GST-ZR7, no phospholipid; lane 10: native α -actinin-1A/GST-ZR7, 25 μ g/ml PI4,5P₂; lane 11: α -actinin-2 ABD-C/GST-ZR7, 600 μ M D-myo-inositol 1,4,5-triphosphate; lane 12: α -actinin-2 ABD-C/GST-ZR7, 600 μ M L- α -glycerophospho-D-myo-inositol 4,5-bisphosphate; lane 13: α -actinin-2 ABD-C/GST-Zq- Δ 6, no phospholipid. (B) Effects of PI4,5P₂ on binding of truncated α -actinin-2 constructs to GST-ZR7. PI4,5P₂, as micelles in 0.5% Triton, was added at 25 μ g/ml in lanes 1–3. Triton micelles alone were used as control in lanes 4–6. Lanes 1 and 4: α -actinin-2 ABD-C; lanes 2 and 5: α -actinin-2 CH2-C; lanes 3 and 6: α -actinin-2 XR1-C.

Pi4,5P₂, similarly to the 2A muscle isoform (Figure 8B). This indicates that the 1A cytoplasmic isoform has a similar structural layout and mode of regulation to the muscle isoform.

Phosphatidylinositol-4-phosphate-5-OH-kinase (PiP-4P-5K) localizes with α -actinin at the sarcomeric Z-disk

The formation of Pi4,5P₂ follows a sequential synthesis pathway involving phosphorylation by dedicated phosphatidylinositol-kinases (Carpenter and Cantley, 1996). Since Pi4,5P₂ underlies a dynamic cellular turnover and is normally of low abundance, we were interested to see whether the enzyme necessary to catalyse the formation of Pi4,5P₂ could be detected in association with the sarcomere at any stage of myofibrillogenesis. Using immunofluorescence detection of the enzyme and confocal microscopy, we can clearly demonstrate that PiP-4P-5K is associated with the sarcomeric Z-disk in neonatal cardiomyocytes (Figure 9). This suggests that Pi4,5P₂ can be effectively formed at these sites and would be available for the activation of α -actinin.

Discussion

The actin-crosslinking protein α -actinin plays a dominant role in the organization of filamentous actin in both non-muscle and muscle cells by crosslinking antiparallel actin

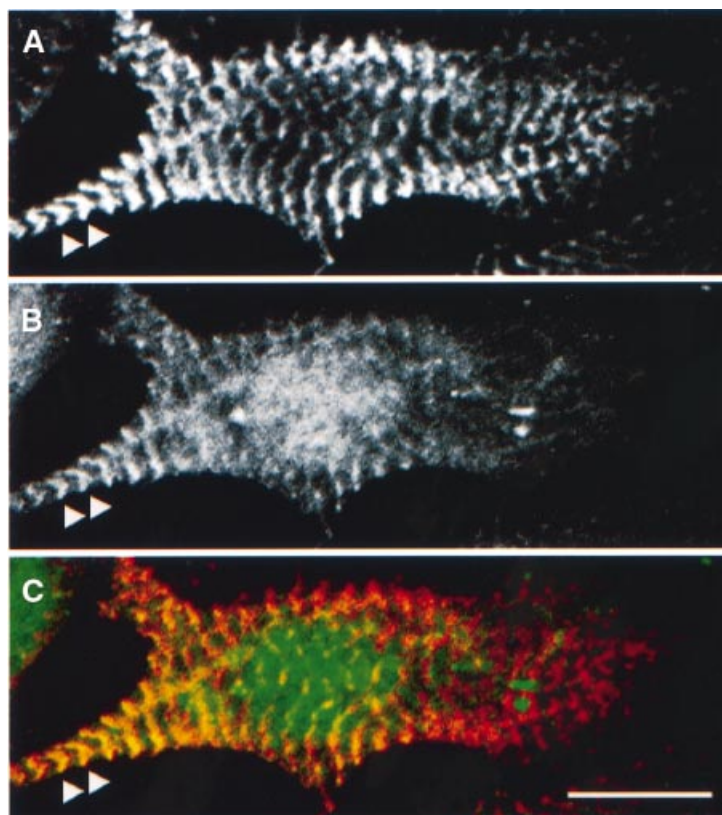


Fig. 9. Localization of PiP-4P-5K in neonatal rat cardiomyocytes. Endogenous proteins were visualized by indirect immunofluorescence using titin T12 to label the Z-disks (A) and a polyclonal antibody labelling PiP-4P-5K (B). Note that PiP-4P-5K is arranged in a striated sarcomeric pattern and the overlay with titin T12 (C) demonstrates its localization close to the Z-band (arrowheads). Note that the sarcomeric association is most pronounced in peripheral regions of the cell and that there is also a significant pool of apparently cytosolic protein. Scale bar, 10 μ m.

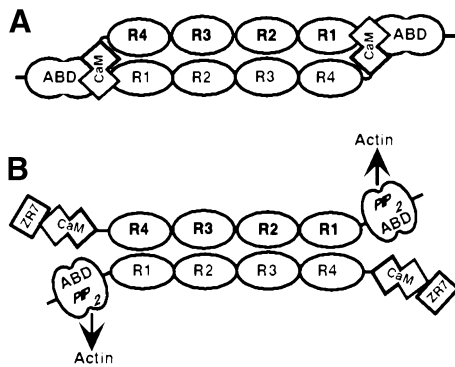


Fig. 10. Model for the regulation of titin Z-repeat binding in α -actinin by phospholipids. (A) The closed or inactive state of the molecule when the EF3/4 region of the CaM-like domain interacts with a region between the ABD and R1 of the opposite subunit. Thus, binding of titin Z-repeats to EF3/4 is prevented. (B) Binding of PI4,5P₂ to the ABD induces a conformational change that switches on titin binding by making the EF3/4 region available for binding to Z-repeats (ZR7). PI4,5P₂ binding also increases the actin-binding properties of α -actinin (Fukami *et al.*, 1992).

filaments into structures of great mechanical stability. The plasticity of the actin-based cytoskeleton requires, however, that local contacts can be quickly remodelled. At sites of membrane anchorage, actin-associated proteins like vinculin can be locally controlled by anionic phospholipids (Niggli and Gimona, 1993; Fukami *et al.*, 1994). The interactions of actin with other proteins, including cytosolic proteins, have been shown to be regulated by PI4,5P₂. These diverse interaction partners include the barbed-end capping protein cap-Z (Heiss and Cooper, 1991; Schafer *et al.*, 1996), the crosslinker α -actinin (Fukami *et al.*, 1992, 1996), the severing protein gelsolin (Hartwig and Yin, 1988) and N1-line titin (Astier *et al.*, 1998). For gelsolin, cap-Z and N1-titin, PI4,5P₂ was reported to inhibit actin interaction. Contrary to that, the interactions of α -actinin with actin (Fukami *et al.*, 1996), and also with several actin-associated proteins like the rho-kinase-related PKN (Mukai *et al.*, 1997), are stimulated by PI4,5P₂. Similarly, the interaction of phosphatidylinositol 3-kinase (PI 3-kinase) with α -actinin was reported to be stimulated by PI4,5P₂ (Shibasaki *et al.*, 1994).

We show here that the interaction of α -actinin with the Z-repeat binding sites on the sarcomeric ruler titin, which is assumed to be responsible for the specific targeting of α -actinin to the sarcomeric Z-disk, is conformationally controlled. A 'closed' conformation of α -actinin is achieved by the interaction of the C-terminal EF-hands 3/4 of one subunit of the antiparallel dimer with a short segment linking the rod to the ABD of the opposite subunit. This closed conformation is relieved upon the interaction of PI4,5P₂ with a site requiring, and probably formed by, both CH domains of the ABD. The PI4,5P₂-bound conformation allows the binding of both F-actin (Fukami *et al.*, 1992) as well as the titin Z-repeats (Figure 10). It has been shown that PI4,5P₂ binding can control the incorporation of α -actinin into the cytoskeleton of non-muscle cells (Fukami *et al.*, 1994). The activation mechanism proposed here would provide an elegant way to coordinate the crosslinking of actin filaments with the targeting of α -actinin to the Z-disk in muscle cells.

This regulatory mechanism requires the spatial proximity of the CaM-like and ABD domains as shown in Figure 10. This is predicted since Ca²⁺ binding to the CaM-like domain regulates actin binding in the non-muscle isoforms. However, there is some contradictory evidence regarding the arrangement of the domains in the α -actinin dimer. Reconstructions based on projection images from two-dimensional crystals have been interpreted as showing the spectrin-like repeats in a staggered arrangement (Taylor and Taylor, 1993); other studies on the dimerization of expressed fragments containing either three or four repeats support the aligned arrangement (Flood *et al.*, 1995, 1997; Figure 1). The two-hybrid binding data presented here, in conjunction with the crystallographic data by Djinovic-Carugo *et al.* (1999), all support the in-phase aligned arrangement. The repeats of α -actinin are thought to be mainly responsible for the dimerization of the molecule, acting as a zipper to bring the ABD and CaM-like domain together (Imamura *et al.*, 1988; Kahana and Gratzer, 1991). Here, we demonstrate for the first time that the CaM domain alone can interact strongly with the opposite subunit in the dimer, independently of the repeats. It is conceivable that the release of these inter-subunit interactions at either end of the antiparallel dimer by PI4,5P₂ binding may result in conformational changes in the rod domain, as this region is expected to be considerably twisted in the free state (Djinovic-Carugo *et al.*, 1999). Such conformational changes could affect the binding of proteins to the rod. In the case of titin, the interaction of zq with the rod domain is constitutive and independent of the phospholipid binding state of the ABD. Like titin, PKN has two interaction sites on α -actinin, one in the CaM-like domain and one in repeat 3 of the rod, and binding is activated by PI4,5P₂. It is possible that the CaM binding site is regulated in a similar manner to titin, but whether the site in repeat 3 is affected by phospholipid binding is unclear. If so, a very long range conformational change in the rod would be required.

The observation that the sarcomeric Z-disk contains PI4,5P₂ (Bullard *et al.*, 1990; Fukami *et al.*, 1992) is puzzling insofar as classical lipid bilayers are absent from the central Z-disk. The nearest lipid compartment in some muscle types are the transverse tubules, which, however, make no direct contacts with the myofibril lattice but are probably anchored by associated intermediate filaments and plectin (Vigoreaux, 1994; Schröder *et al.*, 2000). The major pool of myofibril-associated PI4,5P₂ is therefore likely to be protein bound. Therefore, the lipid moieties of PI4,5P₂ may contribute to α -actinin binding, which might explain why the soluble head group of PI4,5P₂ is unable, at least at relatively low concentrations, to activate α -actinin. A role for the fatty acid moieties of lipids in the regulation of α -actinin has been reported previously for the non-muscle isoform (Burn *et al.*, 1985). Our data suggest that a specific contribution from the lipid moiety might be a common regulatory feature of all α -actinins.

The requirement of PI4,5P₂ for the activation of α -actinin during Z-disk formation suggests that elements of the PI4,5P₂ synthesis pathway may be localized at sites of new sarcomere formation in developing muscle. Indeed, we observe that Pi4P-5K localizes at the Z-disks of neonatal rat cardiomyocytes, which suggests that its

activity in this system is spatially directed to the sites of new sarcomere formation. Remodelling of the actin cytoskeleton upon external stimuli can be achieved by several pathways, of which the small GTPases rho, rac and cdc42 appear to be the most prominent effector molecules (Ridley and Hall, 1992; Ridley, 1996). Rho-GTPases also play major roles as mediators of muscle hypertrophy, but the exact signalling cascades are as yet unknown. In this context, it is interesting to note that the activity of Pi4P-5K is stimulated by rhoA via rho-kinase (Oude Weernink *et al.*, 2000). It will now be interesting to identify the upstream pathways that control phospholipid formation at nascent Z-disks and thus control the integration of new sarcomeres.

Materials and methods

Cloning of cDNA constructs

The cDNA constructs for yeast two-hybrid analysis and protein expression were amplified by PCR. All α -actinin constructs are the human 2A muscle isoform unless otherwise stated. For titin and α -actinin 2A constructs, total human cardiac cDNA (Clontech) was used as the template. For α -actinin 1A constructs, HeLa cell cDNA (Clontech) was used. Primer design was based on the human cardiac titin sequence (DDBJ/EMBL/GenBank accession No. X90568), the human skeletal muscle α -actinin 2 sequence (DDBJ/EMBL/GenBank accession No. M86406) and the human cytoplasmic α -actinin 1 sequence (DDBJ/EMBL/GenBank accession No. X15804). Domain nomenclature for titin is as described in Labeit and Kolmerer (1995) and Sorimachi *et al.* (1997). Phasing of domains for α -actinin constructs was as determined for chicken cytoskeletal α -actinin (Gilmore *et al.*, 1994). All cloning procedures followed standard protocols (Ausubel *et al.*, 1987). The identity of the derived constructs was verified by restriction digest and in some cases by DNA sequencing.

The constructs used (with the amino acid residues in brackets) were as follows. Titin: Z1-ZR3 (1–554); ZR6-Z4 (601–990); ZR7 (644–695); Zq- Δ 6 (695–826). α -actinin 2A: ABD-C (2–894); Δ N-C (19–894); CH2-C (151–894); XR1-C (259–894); R1-C (274–894); XR1 (259–394); R1-R2 (274–501); R2-R3 (371–637); R3-R4 (502–746); ABD-R1 (2–394); ABD-X (2–287); R4-C (627–894); CAM (747–894); EF1/2 (747–820); EF3/4 (821–894). α -actinin 1A: R1-C (267–887).

Yeast two-hybrid analysis

The vectors used for yeast two-hybrid interaction studies were pLexA (Stenmark *et al.*, 1995) and pGAD10 (Clontech). To assay for interactions, cDNA constructs were cloned into these vectors and cotransformed into the *Saccharomyces cerevisiae* L40 reporter strain using a modified lithium acetate protocol (Vojtek *et al.*, 1993). Cells were grown for 2–3 days on synthetic dropout (SD) agar plates lacking the amino acids leucine and tryptophan. Activation of the His3 reporter gene was assayed by restreaking the cells on SD plates lacking histidine. Filter assays for β -galactosidase reporter gene activity were performed as described in the Clontech Matchmaker manual using X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) as colour substrate. Briefly, yeast cells were transferred to genescreen membranes (NEN Life Sciences) and frozen twice in liquid nitrogen to lyse cells. The membrane was then placed on a filter paper soaked in Z buffer (130 mM Na₂HPO₄/NaH₂PO₄ pH 7, 10 mM KCl, 1 mM MgSO₄·7H₂O) plus 0.001% β -mercaptoethanol, 0.85 mg/ml X-gal, and incubated for 6–18 h at 37°C.

Protein expression and purification

All α -actinin constructs were expressed with an N-terminal His₆ tag. The vector used was pET8c-6HTEV, a modified version of the pET 8c vector (Novagen). Expression using this vector produced proteins with the additional amino acid sequence MHHHHHHSTENLYFQGSS at their N-terminus. Digestion of proteins expressed in pET8c-6HTEV with Tobacco Etch Virus (TEV) protease (Gibco-BRL; Parks *et al.*, 1994) removes the His₆ tag and leaves only three additional residues (Gly-Ser-Ser) at the N-terminus of the expressed polypeptide. Expression of the titin constructs was done using pET24d-HisGST-TEV, a modified version of the pET24d vector (Novagen) that expresses proteins with a His₆-tagged GST moiety fused to their N-terminus (gift from G.Stier, EMBL,

Heidelberg). ZR7 was obtained by digesting the HisGST-ZR7 fusion protein to release an untagged polypeptide. All proteins were expressed in *Escherichia coli* strain BL21(DE3). Induction of expression was normally carried out at 37°C using 0.2 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were harvested 4–6 h after induction of expression.

For all recombinantly expressed proteins, initial purification was carried out on a Ni-NTA agarose column (Qiagen). When required, the N-terminal His₆ or His₆-GST tag was removed using TEV protease after the initial Ni-column purification. Cleavage of the tag was performed at 4°C while the protein was being dialysed to 50 mM KH₂PO₄/K₂HPO₄ pH 8, 50 mM NaCl, 20 mM β -mercaptoethanol. The untagged polypeptide was then collected in the unbound fraction from a second Ni-NTA column. Further purification was carried out by ion exchange chromatography on a MonoQ column (Pharmacia). Native Chicken Gizzard α -actinin purified according to the protocol of Langer and Pepe (1980) was a gift from B.Bullard (EMBL, Heidelberg).

GST-column binding assay

Proteins were mixed at a concentration of 8 μ M in 100 μ l of assay buffer (20 mM Tris-Cl pH 7 or 7.5, 50 mM NaCl, 20 mM β -mercaptoethanol) and incubated for 30 min on ice. They were then loaded on a mini column packed with 50 μ l of glutathione-Sephrose (Stratagene) and pre-equilibrated with assay buffer. The column was washed once with 50 μ l and twice with 100 μ l of assay buffer and bound proteins were then eluted in 100 μ l of Laemmli sample loading buffer (62 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.001% Bromophenol Blue). The eluted proteins were analysed on 12% SDS-PAGE gels (Laemmli, 1970). Assays involving phospholipids or phospholipid head groups were performed at pH 7 in the same way. Phospholipids (Sigma) were prepared at 1 mg/ml, as micelles, by sonication in a solution containing 0.5% Triton X-100, 20 mM HEPES pH 7, 0.1 mM EDTA. D-myo-inositol 1,4,5-triphosphate (Alexis) and L- α -glycerophospho-D-myo-inositol 4,5-bisphosphate (Sigma) were prepared at 1 mg/ml directly in assay buffer. Phospholipids or phospholipid head groups were diluted to the desired final concentration in assay buffer and this was used to dilute the proteins as well as equilibrate and wash the column.

Ni-column binding assay

α -actinin CaM-like domain and His₆-tagged ABD-R1 or ABD-X were mixed at 20 μ M each in a total volume of 100 μ l binding buffer (20 mM K₂HPO₄/KH₂PO₄ pH 8, 50 mM NaCl, 5 mM imidazole, 20 mM β -mercaptoethanol) and incubated for 30 min on ice. They were loaded on a mini column packed with 50 μ l of Ni-NTA agarose (Qiagen). The column was washed with 3 \times 100 μ l of binding buffer and bound proteins were then eluted in 100 μ l of elution Laemmli sample loading buffer. The flow-through and wash, as well as the eluted proteins were collected and 20 μ l aliquots were analysed on 15% SDS-PAGE gels (Laemmli, 1970). In competition experiments, titin-ZR7 or the α -actinin peptide (amino acids 259–287) was added at various concentrations prior to incubation of proteins on ice. The peptide was purchased from Interactiva.

Isothermal calorimetry

Isothermal calorimetry was performed using the VP-ITC calorimeter from Microcal, USA. Proteins were prepared by dialysing simultaneously overnight in ITC buffer (20 mM MES pH 7.0, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM Na₃N₃). After concentration and just prior to use, proteins were centrifuged for 15 min at 15 000 r.p.m. in an Eppendorf centrifuge and degassed for 10 min with stirring. All experiments were performed at 26°C with stirring at 350 r.p.m. The α -actinin CaM-like domain or R4-C was injected into the experimental cell, which contained ZR7 or ABD-R1 at a 10–20-fold lower concentration. The reference cell contained water and 1 mM Na₃N₃. Injection volumes of 8–10 μ l were used, with an initial small injection of 2 μ l. Reference data for the heat of dilution of the injectant were determined by performing an identical experiment with only buffer in the experimental cell. Raw data were normalized per mole of injectant and integrated with respect to time, and then reference data were subtracted. Data were fitted by least squares minimization using the Origin software package (Microcal, USA) to optimize the fitting parameters stoichiometry (*n*), enthalpy (Δ H) and association constant (*K*_b). A single binding site model was used to fit the data in all cases, and gave the best r² values.

Cell culture and immunofluorescence

Neonatal rat cardiomyocytes were isolated from day-3 Wistar rats as described previously (Sen *et al.*, 1988; Komiyama *et al.*, 1996). The cells were plated on collagen-coated 35 mm dishes and grown in M199 medium, 5% fetal bovine serum, 5% horse serum, 10 μ M cytosine-

arabinoside and 10 μ M phenylephrine for 3 days at 37°C. Cells were fixed in 2% paraformaldehyde on ice and processed for immunofluorescence microscopy following standard methods. Z-disk titin was stained with the monoclonal antibody T12, phosphatidylinositol-4-phosphate-5-kinase with a goat polyclonal antibody (SantaCruz Biotechnology). Bound antibodies were visualized with compatible anti-mouse-alexa546 and anti-goat-alexa488 antibody conjugates (Molecular Probes). Confocal microscopy was carried out using a BioRad 400 confocal microscope.

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